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Quantifying proteomes and their post-translational modifications by stable isotope label-based mass spectrometry

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Abstract

Stable isotope labeling coupled with mass spectrometry has revolutionized the scope and impact of protein expression studies. Label incorporation can occur metabolically or chemically, and each method bears specific strengths and weaknesses. Quantitative proteomics confidently identifies specific interactions between proteins and other biological species, such as nucleic acids and metabolites. Extending label-based methods to phosphorylation-modified forms of proteins enables the construction of signaling networks and their temporal responses to stimuli. The integration of multiple data types offers systems-level insight on coordinated biological processes. Finally, the development of methods applicable to tissue quantification suggests the emerging role of label-based, quantitative mass spectrometry in translational science.

Introduction

Mass spectrometry (MS) has become the central technology for large-scale protein identification studies. Though touted for its sensitivity and throughput, MS is not inherently quantitative, an increasing requirement needed to fuel the emergent systems and quantitative biology paradigms. For decades mass spectrometrists have used stable isotope-labeled internal standards to quantify small molecules, and in recent years, proteomic researchers have applied the same tactics, with a few twists, to measure up the proteome. Among the first global proteome quantification technologies was the isotope coded affinity tagging (iCAT) method pioneered by Gygi, Aebersold, and co-workers [1]. iCAT is a chemical label that places heavy or light tags on cysteine side chains. This pioneering work was expanded upon in 2003 when Thompson et al. described more broadly versatile and isotopically complex chemical reagents that offered multiplexed proteome quantification (i.e., tandem mass tags, TMT) [2]. The very next year Pappin and colleagues published on their own version of the isobaric tag: isobaric tags for relative and absolute quantification (iTRAQ) [3]. At about the same time Mann and others incorporated stable isotopes metabolically by growing cells on isotopically-labeled amino acids – stable isotope labeling by amino acids in cell culture (SILAC) [4,5]. These and other technical innovations have ignited the field of

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quantitative proteomics so that thousands of biological experiments – ranging from basic to translational – are now conducted each year. In this review we highlight new developments in these label-based strategies, how they are applied to a diverse array of biological problems, and the integration of these data with other systems-level analyses.

Advances and challenges in label-based quantification strategies

A growing trend in proteome quantification is the demand for parallel sample processing – i.e., multiplexing. MS experiments often require days of constant instrument operation to achieve good proteomic depth, meaning that comparing dozens, let alone, hundreds of samples can become prohibitive. Isobaric tagging approaches currently present the most straight-forward path to multi-plexed (up to 8 samples) proteome analysis by concealing quantitative information until tandem MS (MS/MS) (Figure 1, left) [6–8]. Simultaneous analysis of multiple proteomes allows the comparison of not only numerous conditions, but also biological replicates within the same experiment [9•]. In this fashion, rigorous statistical analysis can be performed so that quantitative false discovery rates can be imposed – a real confidence builder when selecting targets for follow-up biology. Isobaric tagging, however, does have two notable limitations: first, an MS/MS scan is required for quantification so that, from experiment to experiment, there can be low overlap in which peptides are detected, and, therefore, quantified; second, multiple precursors are almost always co-isolated and fragmented during MS/MS. The result is contamination of the reporter ion signal from co-isolated impurities, which can significantly erode quantitative accuracy [10,11].

Non-isobaric methods, such as SILAC, iCAT, reductive dimethylation, ^{15}N , etc., avoid the aforementioned problems by exposing quantitative information in high resolution MS^1 scans, where interfering species are readily distinguished (Figure 1, right). That said, all of these MS^1 -based techniques have greatly limited plexing capacities (up to ~ 3), because each labeled version creates a new isotopic cluster for a given peptide. A minimum of 4 Da between each cluster is needed to keep the clusters from overlapping. Thus, a three-plex SILAC experiment generates a proteome with triple the complexity, which, in turn, lowers sampling depth and, subsequently, proteomic coverage. Consequently, a single SILAC experiment generally compares two or three proteomes at a time, and higher-order comparisons are obtained by interlacing multiple like-experiments [12]. Recently, we described a new method that synthesizes the positive attributes of both isobaric tagging and SILAC, while mitigating the downsides. The approach, called neutron encoding (NeuCode), exploits the subtle (~ 6 mDa) mass defects in common stable isotopes to expand SILAC multi-plexing without the increase in spectral complexity that accompanies traditional SILAC approaches [13•]. Briefly, amino acid isotopologues differing in mass by as little as 6 mDa are incorporated during protein synthesis and resolved by high resolution ($>200,000$) mass spectrometry to enable quantification. This fresh SILAC variant offers the potential to achieve a quantitative capacity on par with isobaric labeling, while maintaining the measurement accuracy characteristic of MS^1 -based methods.

In sum, stable isotope incorporation methods are considered proteome quantification gold standards and are more approachable today than ever. Our ability to achieve highly plexed proteome quantification with excellent proteomic depth and quantitative accuracy continues to evolve. In the future, look to hybrid approaches, such as NeuCode [13•] and hyperplexing [9•], to bridge the current isobaric tagging and SILAC technologies. For the remainder of this review we will highlight recent noteworthy applications of these technologies: identifying physical interactions, elucidating dynamic signals, and enabling true systems-level analyses.

Identifying physical interactions

Affinity purification, coupled with mass spectrometry (AP-MS), has long been used to interrogate the nature of interactions between proteins of interest and other proteins in the surrounding environment, but this approach has been limited by the need for stringent purification to reduce background binding. While eliminating proteins that bind ubiquitously, these rigorous washings also remove weakly binding, but genuine interacting, proteins as well. Label-free strategies for characterizing protein complexes have increased in robustness, utilizing probabilistic scoring systems to indirectly quantify protein-protein interactions [14,15]. Alternatively, quantitative proteomics can directly distinguish specific from non-specific interactions [16]. Using SILAC, for example, if light-labeled cells are incubated with control bait, and heavy-labeled cells with prey bait, proteins that exhibit a quantitative ratio that reproducibly deviates from 1:1 (the quantitative ratio for background proteins that equally bind the control and prey baits) are likely specific interactors.

The use of this quantitative AP-MS strategy has expanded in recent years. Specifically, it has been applied to study the temporal specificities of protein complexes that drive central processes, such as orchestrated progression through the cell cycle by the conserved cyclin-cdk machinery [17]. When conducted in parallel with phosphoproteome quantification, phase-specific interaction dynamics can be further detailed, as demonstrated by the time-of-day specificities revealed in the circadian clock of *Neurospora crassa* [18]. Triple SILAC labeling (i.e., light-, medium-, and heavy-labeled cells) affords ternary comparisons for fine-tuning the specificities of protein-protein interactions. Such an approach has determined stimulus-specific interactions in the Wnt pathway [19], effects of combinatorial tyrosine phosphorylation on insulin receptor binding [20], and crosstalk between histone modifications [21].

In addition to elucidating protein-protein interaction networks, quantitative AP-MS also improves confident identification of protein complexes involving other types of biological molecules. DNA-protein [22] and RNA-protein [23] interaction screens have been developed to study sequence-specific binding patterns. An especially interesting application of this technology allows for rapid screening of single nucleotide polymorphisms (SNPs) associated with type 1 diabetes for differential transcription factor binding [24•]; this tool, when combined with the SNP mapping capabilities of genome-wide association studies (GWAS), strengthens evidence supporting the link between a given variant and a common disease. Recently, cholesterol-protein interactions were mapped in living cells using photoreactive sterol probes and SILAC quantification [25••]. The diversity of protein interaction partners already investigated by quantitative proteomics illuminates the feasibility of constructing global protein, nucleic acid, and metabolite interaction maps and the utility of these comprehensive interactomes to systems biology.

Elucidating dynamic signals

Advances in sample preparation, instrumentation, and informatics have fueled the popularization of global and targeted studies of protein phosphorylation. Compared to a decade ago, our understanding of, and ability to perform phosphopeptide enrichment, quantify phosphorylation events, localize sites to specific residues, and build signaling networks from phosphoproteomic datasets, has greatly progressed [26,27].

As coordinators of the placement and removal of phosphorylation events, and the timing of such actions, kinases and phosphatases are the gatekeepers of signal transduction. In fact, entire families of kinases are differentially activated in distinct cell types, which highlights the level of biological control they exert (Figure 2). It is not surprising then that they have come to serve as key targets in mass spectrometry-based quantitative phosphoproteomics

experiments. Many studies elucidate signaling networks by abolishing kinase or phosphatase activity, either through small molecule inhibitors or by genetic knockdown, and quantifying how the phosphoproteome responds to a relevant stimulus. Recent work by multiple researchers has focused on characterizing substrates of mTORC1, a Ser-Thr kinase complex that plays a pivotal role in controlling cell growth and metabolism; applying quantitative phosphoproteomics to track downstream effectors generates potential targets for cancer therapy [28••] and connects this pathway to previously unassociated biological processes [29]. Other analyses profile kinome phosphorylation directly via kinase enrichment using inhibitors as immobilized capture ligands, a promising route to access proteins of low cellular abundance that are often underrepresented in quantitative mass spectrometry experiments [30].

As the scale of phosphoproteomics experiments continues to increase, the importance of data interpretation becomes undeniable. Determining differential phosphorylation events requires untangling changes in phosphorylation at a given site on a protein from changes in that protein's expression. Calibrating phosphopeptide ratios by their corresponding protein levels maximizes the accuracy with which the captured snapshot of differentially regulated phosphorylation events represents the biological system under investigation [31•]. While relative phosphoproteome quantification has become routine, the calculation of absolute phosphorylation site stoichiometries remains a challenge. The functional role of protein phosphorylation is dictated not only by its presence or absence, but also by its fractional occupancy at a given site. A few methodological [32] and computational [12] strategies have been proposed, but have yet to be incorporated into the standard toolbox of methods for global phosphoproteome quantification.

Enabling true systems-level analyses

As the robustness and throughput of protein quantification technologies continue to mature, integrating proteome- and genome-level measurements is becoming not only achievable, but also necessary for understanding essential biological phenomena [33,34]. Understanding the coordinated nature of these processes often has a therapeutic benefit. For example, alterations in the primary genome, such as amplifications or deletions, can be associated with known tumor promoting activities; thus, linking chromosomal aberrations with their functional consequences at the protein level is of interest to the cancer genomics community [35]. Other processes dictate routes for microbial engineering that could revolutionize biofuel production; for example, pinpointing the factors limiting efficient biomass conversion in model microbes generates target enzymes for manipulation in engineered microbes [36]. It is clear that the ability to integrate multiple data types will impact medicine and environmental science in ways unimaginable with a single approach.

Important biological events, however, are not always defined by a drastic remodeling of genes, transcripts, and proteins. Hence, replicate experiments, in addition to multiple levels of analysis, are needed to increase statistical power and confident detection of subtle expression changes. An illustration of the biological insight afforded by such an experimental design is a recent transcriptomic, proteomic, and phosphoproteomic comparison of four human embryonic stem (ES) cell lines with four induced pluripotent stem (iPS) cell lines, in biological triplicate [37•]. Minor deviations between ES and iPS cells were detected at all three levels of regulation, but only through biological replicate analysis, which enabled the determination of differential regulation by statistical significance rather than arbitrary fold-changes. In fact, greater than 90% of the differentially regulated transcripts, proteins, and phosphorylation sites differed by less than two-fold, emphasizing the blindness of single-replicate approaches to subtle, yet reproducible differences in expression.

A major goal of systems biology that still remains in its infancy is the construction of mathematical models that accurately represent and predict cellular events and outcomes. Comprehensive models of gene expression in yeast [38] and murine [39••] systems serve as blueprints for what is expected to become a widely incorporated analysis.

Translating from cell culture to disease models

The development and application of label-based methods have predominantly taken place in cultured cells, in part because of their direct amenability to both chemical and metabolic labeling. To equip quantitative proteomics for a greater translational impact, current methods must be extended to animal disease models and, ultimately, to human tissue proteomes.

So far, label free and chemical labeling approaches have been used in global phosphorylation and acetylation studies to construct tissue-specific maps [40–42] and to investigate perturbations to cell metabolism [43,44]. Applying metabolic strategies to tissue analysis is not as straightforward because label incorporation during protein synthesis is needed, although metabolic labeling has been accomplished in a variety of non-human animals [45]. The SILAC mouse was first introduced five years ago [46] and has since provided tissue for *in vivo* quantitative studies on aging [47] and cardiac [48] proteomics.

For obvious reasons, human tissue, the ultimate system for studying relevant diseases, is challenging to metabolically label. To circumvent this limitation, a mixture of multiple SILAC-labeled cell lines that accurately represents the tissue can serve as a robust internal standard for tissue quantification, a method termed super-SILAC [49]. This strategy effectively decouples proteomic analysis from the biological experiment, expanding the quantitative scope of SILAC labeling approaches. Though it has yet to be widely adopted for the study of human tissue, super-SILAC has been used to classify cell lines derived from patients with various lymphoma subtypes [50] and adapted for global phosphosite quantification in tissues [51•].

Conclusions and outlook

The next generation of proteomics will rely heavily on continued advances in mass spectrometry technologies [52•]: expanded multi-plexing capacity of quantitative methods will enable more sample comparisons in shorter amounts of time, intelligent data acquisition schemes will improve quantification quality and reproducibility between multiple experiments [53], and the diversity and combinatorial effects of protein post-translational modifications, such as acetylation [42,44] and ubiquitylation [54,55], will be uncovered. Most importantly, large-scale studies of protein expression will be integrated with other systems-level approaches to provide a more holistic perspective on biological processes key to sustaining the health of the planet and its inhabitants.

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Highlights

- Stable isotopes enable accurate and multi-plexed proteome quantification by MS
- Quantitative strategies can elucidate physical interactions and signaling networks
- Integrative approaches profile multiple stages of gene expression regulation
- Quantitative MS is becoming increasingly equipped to impact translational science

Figure 1.

Stable isotope incorporation strategies for relative proteome quantification by mass spectrometry. Two predominant methods for label-based quantification are isobaric tagging and SILAC. Isobaric tags label the N-termini and lysine residues of peptides resulting from protein extraction and proteolytic digestion. All labels in a set bear the same intact mass, meaning that peptides from differentially-labeled samples will appear at the same precursor m/z . Only upon fragmentation are the reporter ions released to permit quantification. Alternatively, in metabolic approaches such as SILAC, heavy isotope-labeled amino acids are incorporated during protein synthesis. Samples can be mixed together prior to cell lysis so that all downstream processing steps are performed on all samples simultaneously. SILAC-labeled peptides are quantified from full MS scans across the elution profile.

Figure 2. Cell type-specific activation of kinase families. A phosphoproteomic comparison of human embryonic stem (ES) cells with human fibroblast (newborn foreskin fibroblast, NFF) cells revealed a pool of phosphorylation sites that was differentially regulated between the cell lines. These phosphorylation sites serve as kinase substrates whose cognate protein kinases can be predicted computationally. The activation of entire families of kinases can depend on cell type. For example, CMGC kinases are highly-activated in ES cells, while CAMK and AGC kinase substrates are enriched in NFF cells.